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A major ozonation product of cholesterol, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, induces apoptosis in H9c2 cardiomyoblasts

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Abstract Cholesterol, a major neutral lipid component of biological membranes and the lung epithelial lining fluids, is susceptible to oxidation by reactive oxygen and nitrogen species including ozone. The oxidation by ozone in biological environments results in the formation of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (cholesterol secoaldehyde or CSeco, major product) along with some other minor products. Recently, CSeco has been implicated in the pathogenesis of atherosclerosis and Alzheimer's disease. In this communication, we report that CSeco induces cytotoxicity in H9c2 cardiomyoblasts with an IC_{50} of 8.9 ± 1.29 μM (n = 6). The observed effect of CSeco at low micromolar concentrations retained several key features of apoptosis, such as changes in nuclear morphology, phosphatidylserine externalization, DNA fragmentation, and caspase 3/7 activity. Treatment of cardiomyocytes with 5 μM CSeco for 24 h, for instance, resulted in 30.8 ± 3.28% apoptotic and 1.8 ± 1.11% of necrotic cells as against DMSO controls that only showed 1.3 ± 0.33% of apoptosis and 1.6 ± 0.67% of necrosis. In general, the loss of cellular viability paralleled the increased occurrence of apoptotic cells in various CSeco treatments. This study, for the first time, demonstrates the induction of apoptotic cell death in cardiomyocytes by a cholesterol ozonation product, implying a role for ozone in myocardial injury.

Keywords: Cholesterol secoaldehyde; Apoptosis; Cardiomyocyte; Ozone

1. Introduction

Cholesterol is one of the most abundant neutral lipids in the biological membranes and is a component of pulmonary surfactant [1]. The $\Delta^{5,6}$ double bond in cholesterol is susceptible to oxidation by reactive oxygen and nitrogen species including ozone [2]. The reaction with ozone has been shown to form several products including a major product, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al [cholesterol secoaldehyde (CSeco) (Fig. 1)]. CSeco has been detected in lung homogenates and bronchoalveolar lavage fluid of rats exposed to ozone [3,4], and has in fact been suggested as a biomarker for ozone exposure [5]. A recent report by Wentworth et al. [6] demonstrates the formation of CSeco in arterial plaques following treatment with phorbol esters indicating the production of endogenous oxidants under inflammatory conditions. CSeco formed in reactions of ozone with cholesterol in lung surfactant has been shown to be cytotoxic to human bronchial epithelial cells [7]. Other oxysterols similar to CSeco but produced in autoxidation reactions have been shown to be cytotoxic to numerous cell types [8] and have been known to play an important role in the development of cardiovascular diseases [9]. The relation between ozone and myocardial diseases has been reported in several epidemiological studies [10,11]. Reports concerning the cytotoxicity of CSeco effecting the cardiovascular system have however been limiting. Therefore in the present study, experiments were conducted to delineate the effects of CSeco on cardiomyocytes. It was observed that CSeco induced apoptotic cell death at low micromolar concentrations. The apoptosis increased in a dose-dependant manner indicating that CSeco may probably be involved in myocardial injury, and is reported in the present communication.

2. Materials and methods

2.1. Synthesis of CSeco

The synthesis of CSeco was performed according to the method of Wang et al. [3]. Briefly, 1 mmol of cholesterol (>99%; Sigma) was dissolved in 150 ml of dichloromethane/methanol (1/1, v/v). A gentle stream of ozone (4 mmol/h) in oxygen (300 ml/min), generated by Sander 200 ozonizer (Erwin Sander, Uetze-Eltze, Germany), was allowed to bubble through the cholesterol solution until ⩾ 99% of cholesterol was oxidized. The extent of oxidation of cholesterol was assessed by reversed phase (RP) HPLC on a Discovery HS C18 column (4.6 mm × 250 mm) using a mobile phase that contained 40% isopropanol in acetonitrile at a flow of 1 ml/min (the eluent was monitored at 210 nm). Upon verification that cholesterol was completely oxidized, the sample was reduced with Zn/AcOH and purified by solvent extraction [3]. The purity of the final product was tested by RP-HPLC. Stock solutions of the final product were prepared in DMSO at a concentration of 20 mM and stored in small aliquots at −80 °C until use.

2.2. Cell culture

Rat heart cell line H9c2 was obtained from ATCC (Rockville, MD). The cells were cultured at 37 ± 0.5 °C in 5% CO_2 in DMEM containing 4 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, 10% FBS, 100 U/ml penicillin, and 100 μg/ml

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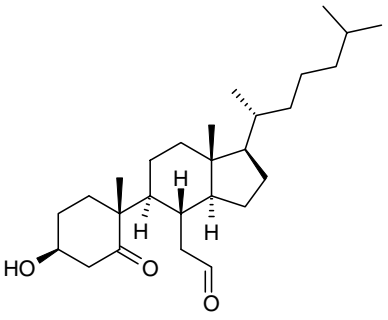

Fig. 1. Structure of CSeco.

streptomycin. The medium was changed once in every 2–3 days. To prevent the loss of differentiation potential, the cells were not allowed to become confluent. The cells at ca. 70% confluence were exposed to varying concentrations of CSeco in a medium that contained 2% FBS and antibiotics. Care was taken to see that the final concentration of DMSO in the control as well as cell cultures exposed to CSeco was always $\leqslant$0.01% (v/v).

2.3. Cell viability

A non-radioactive assay, CellTiter 96 AQ_{ueous} from Promega (Madison, WI) was used to assess cell viability or proliferation. The assay was based on the reduction of a water-soluble tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by viable cells in a phenazine methosulfate (PMS)-assisted reaction. The H9c2 cultures (500 μl each) in DMEM were seeded in 24-well culture plates (Corning, Acton, MA) at 3×10^4 cells/well. The medium was replaced with DMEM containing 2% FBS, antibiotics, and varying concentrations of CSeco (0–25 μM) and the incubation continued for 24 h. At the end of the incubation period, an aliquot (20 μl) of the MTS/PMS reagent (20:1) supplied by the manufacturer was added per well. The cells were incubated for 4 h and the MTS formazan produced was measured at 490 nm using a Biotek EL 800 microplate reader. Background absorbance from wells that contained CSeco in DMEM but no added cells was subtracted. The viability of cells not exposed to CSeco was set to 100% and the viability in CSeco-treated cultures was expressed relative to the controls.

2.4. Release of cytosolic lactate dehydrogenase

Release of cytoplasmic LDH is a means of measuring membrane integrity. We performed this assay in cardiomyocytes exposed to CSeco using CytoTox-ONE homogeneous membrane integrity kit from Promega (Madison, WI). After exposing cardiomyocytes to different concentrations of CSeco (0–25 μM) for 24 h, the culture plates were centrifuged at $250 \times g$ for 4 min. An aliquot (100 μl) of the supernatant was transferred to a clean, flat-bottomed black well plate and 100 μl CytoTox-ONE reagent was added. The plates were protected from light and incubated at room temperature for 10 min. The fluorescent signal was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The LDH release is expressed as percentage of total LDH (i.e., following complete lysis of cells).

2.5. Morphological staining using acridine orange and ethidium bromide

Acridine orange (AO) stains DNA bright green and, therefore, allows visualization of chromatin pattern in apoptotic and necrotic cells. Ethidium bromide (EB) stains DNA orange but is excluded by viable cells. Dual staining with AO-EB thus allows enumeration of populations of cells that are viable-non-apoptotic, viable-apoptotic, nonviable-apoptotic, and necrotic. We performed the AO-EB assay in cardiomycytes exposed to CSeco as described by Agrawal et al. [12]. Briefly, cardiomyocytes treated with CSeco (5 and 10 μM) for 24 h were trypsinized, and cell pellets were collected by centrifugation at $300 \times g$, resuspended in 50 μl of complete medium and stained with 4 μl of AO-EB working solution (AO: 100 μg/ml; EB: 100 μg/ml) in 0.9% saline. Samples were analyzed immediately by fluorescence microscopy (excitation: 488 nm; emission: 520 nm). Percentage of total apoptosis was obtained from the sum of viable apoptotic and nonviable apoptotic cells divided by the total number of cells.

2.6. DNA fragmentation

Cardiomyocytes in 24-multiwell plates were treated with various concentrations of CSeco for 24 h. The cytoplasmic histone-associated DNA fragments, which are indicative of apoptosis, were measured using sandwich ELISA (Cell Death Detection kit, Roche; Indianapolis, IN). Briefly, cells were lysed and the lysate was added to streptavidin-coated 96-well plates. A mixture of biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies was added, incubation continued for 2 h at room temperature, and then washed. The amount of cytoplasmic nucleosome was quantified based on the formation of 2,3-diaminophenazine at 405 nm. The relative yields of apoptosis were determined based on the ratio of absorbance in the wells with CSeco-treated cells versus those with control, untreated cells. Results were averaged from three different experiments performed in duplicate.

2.7. Caspase-3/7 protease activity

Cardiomyocytes in 24 well plates were treated with 0–25 μM CSeco for 24 h. Upon lysis of cardiomyocytes, the activity of caspase 3/7 was determined based on proteolytic cleavage of rhodamine 110, bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) (Apo-ONE® Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). The sequential cleavage and removal of

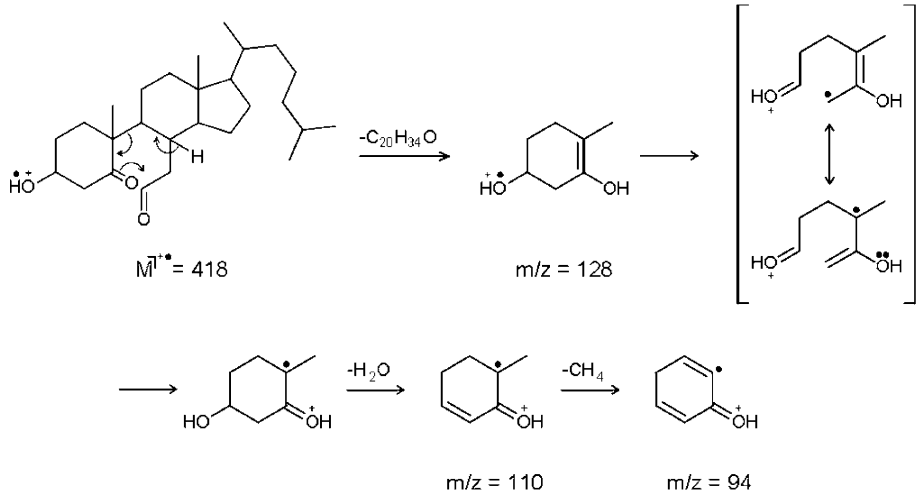

Fig. 2. Structure and fragmentation pattern of the molecular ion of CSeco formed in GC/MS/EI.

the DEVD peptides by caspase 3/7 results in the formation of free rhodamine 110, which is quantified using Spectermax Gemini fluorescence spectrophotometer (excitation: 499 nm; excitation: 521 nm). Caspase 3/7 activity is expressed as number of folds increase relative to untreated control.

2.8. Annexin-Vlpropidium iodide assay

Phosphatidylserine (PS) translocation resulting in loss of plasma membrane asymmetry and changes in nuclear morphology, that are characteristic of apoptotic process, were performed using annexin-V-FLUOS staining kit (Roche, Indianapolis, IN). Cardiomyocytes grown to subconfluency on cover slips were treated with different concentrations of CSeco for 24 h. The cells were then incubated with annexin-V-FITC conjugate and propidium iodide for 15 min at room temperature in a buffer that facilitates binding. At the end of incubation, digital images were taken using a Nikon E400 fluorescence microscope and image capture were performed using SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). Cells were examined for changes in the plasma membrane and nuclear morphology and were compared with different treatments to qualitatively confirm apoptosis.

2.9. Statistical analysis

Values of IC_{50} were calculated by fitting the log concentration–response curves by standard non-linear regression analysis using Prism 4.0 (GraphPad Software, San Diego, CA). All data are expressed as means ± SEM from three or more independent experiments.

3. Results

3.1. Synthesis of cholesterol secoaldehyde

A convenient method for the synthesis of CSeco, described by Wang et al. [3], involves ozonation of cholesterol in a

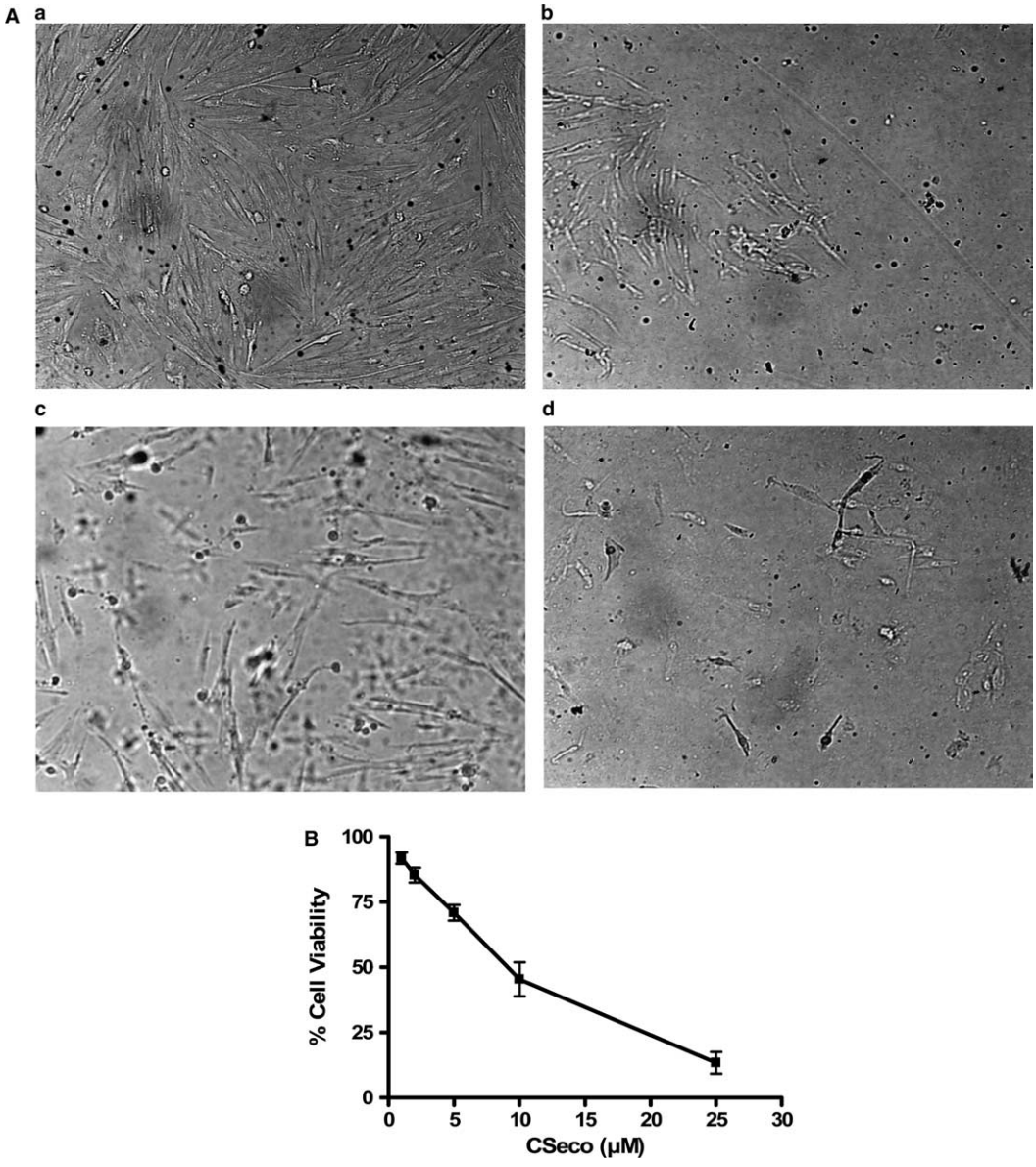

Fig. 3. Effect of CSeco on cardiomyocytes. (A) Induction of morphologic changes and cell death in cardiomyocytes treated with CSeco at (a) none (control), (b) 5 μM, (c) 10 μM and (d) 25 μM. Cardiomyocytes were examined by phase-contrast microscopy after 24 h of CSeco treatment. (B) CSeco exposure decreases cellular viability of cardiomyocyes in the MTS reduction assay. Cells were treated with different concentrations of CSeco for 24 h. Cell viability measured using CellTiter 96 AQ_{ueous} kit.

participating solvent followed by reduction of the product mixture with Zn/AcOH. We have used a similar methodology but employed RP-HPLC instead of TLC to monitor the progress of ozonation. The use of RP-HPLC allows a rapid and accurate monitoring of unreacted cholesterol in the ozonation mixtures. In addition to carbon–carbon double bounds, ozone is known to react with carbon–hydrogen bounds in aliphatic hydrocarbons with the selectivity being more for tertiary than for secondary and primary alkanes [13]. The second-order rate constants for the reaction of ozone with carbon–carbon double bonds, in general, are several orders of magnitude higher than for carbon–hydrogen bonds [13]. Therefore, the oxidation of C–H bonds of cholesterol that is likely to occur at C8, C9, C13, C17, C20, and C25 positions can be minimized by controlled ozonation. We performed high resolution mass spectral analysis as well as GC/MS/EI fragmentation on CSeco synthesized. The exact mass was 418.340692 which compares well with the expected value of 418.344696. The GC/MI/EI fragmentation showed a molecular ion (M^+) at *m*/*z* 418, and daughter fragments at *m*/*z* 128, 110, and 93. The fragmentation pattern is outlined schematically in Fig. 2.

3.2. CSeco is a potent inducer of cytotoxicity in cardiomyocytes

The MTS reduction assay shown in Fig. 3A indicates a marked induction of cell death in cardiomyocytes treated with varying low concentrations of CSeco for 24 h. The concentration of CSeco required for 50% cell death was calculated from the dose–response curve. It was found to be 8.9 ± 1.29 μM ($n = 6$). Microscopic evaluations of cell cultures revealed a significant loss of cell survival at each concentration of CSeco. Also, there were major morphological changes such as cell shrinkage and membrane blebbing (Fig. 3B).

3.3. Cytosolic LDH release

CSeco induces LDH release to the extracellular medium only at concentrations $\geqslant 25$ μM (Fig. 4). After 24 h of exposure, doses of 1–10 μM CSeco did not induce any significant LDH release. This confirms that CSeco does not induce changes in cellular integrity at concentrations lower than 10 μM.

3.4. Cholesterol secoaldehyde induces apoptosis in cardiomyocytes

The images of cardiomyocytes shown in Fig. 5A were obtained after staining with AO and EB. Treatment with CSeco at concentrations of 5 and 10 μM for 24 h resulted in higher proportion of cells with dying nuclei than in control cells. At 5 μM CSeco, there were 30.8 ± 3.28 apoptotic and 1.8 ± 1.11% necrotic cells as against DMSO controls that only showed 1.3 ± 0.33% of apoptosis and 1.6 ± 0.67% of necrosis. The % apoptotic and necrotic cells at 10 μM CSeco were 56.9 ± 5.97 and 3.4 ± 0.94, respectively (Fig. 5B). The condensed chromatin that stained uniformly and the cellular integrity indicated that the cell death is typical of apoptosis.

We used three different assays to further confirm the occurrence of apoptosis. These include DNA fragmentation, caspase 3/7 activity, and staining with annexin-V/propidium iodide. Treatment of cardiomyocytes with CSeco resulted in a dose-dependent increase in the histone associated cytoplasmic DNA fragments, with the highest level being observed around 10 μM CSeco. There was less DNA fragmentation, but increased cell death at concentrations of CSeco $\geqslant 25$ μM, indicating a change from apoptotic to necrotic cell death at higher concentrations (Fig. 6). It was found that CSeco concentrations of 1–10 μM which promoted DNA fragmentation also induced a dose-dependent activation of caspase 3/7, with maximal activity observed at 10 μM CSeco (Fig. 7). There was a significant loss of plasma membrane asymmetry due to phosphatidylserine translocation in cardiomyocytes treated with 5 and 10 μM CSeco for 24 h but not in the control cells (Fig. 8).

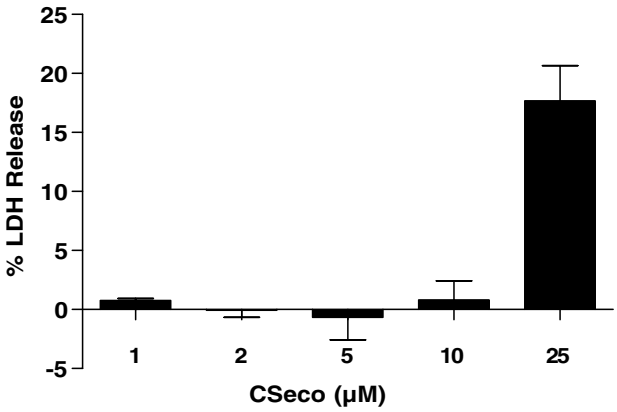

Fig. 4. Effect of CSeco on LDH release. Cardiomyocytes were incubated with the indicated CSeco concentrations for 24 h. Results are expressed as percentages of LDH release after subtracting the control values. Data are means ± SEM of four separate experiments from cells of different cultures, each one performed in triplicate.

4. Discussion

The major finding of this study is that CSeco, a cholesterol ozonation product induces a dose-dependent apoptosis in H9c2 cardiomyoblasts. The mechanism of apoptosis is not clear; however, in analogy with other oxysterols, the apoptotic process could involve derangement in cholesterol homeostasis [14], alterations in cell-cycle regulation [15], and/or changes in inflammatory signaling [16].

The cytotoxic effects of CSeco may have a clinical implication, especially in the light of recent reports demonstrating the occurrence of CSeco in the atherosclerotic plaques [6] and in brain samples of patients with Alzheimer's disease [17]. Wentworth and his coworkers detect 60 pmol of CSeco per mg of human atherosclerotic plaque [6]. Assuming an approximate density of 1 g/ml, the concentration of CSeco in the lesion material would equate to 60 μM. This concentration is about one-order of magnitude higher than the IC_{50} reported in the present study, meaning that cholesterol ozonation products may increase the risk of myocardial injury.

The biochemical origin as well as the metabolic fate of 'ozone-specific' oxysterols is largely unknown. Babior et al. [18] and Wenworth et al. [6] suggest that ozone produced at the inflammatory sites reacts with cholesterol resulting in the formation of CSeco. The other possibility is that ozone in the breathing air oxidizes cholesterol at the air/lung interface and the products formed are then transported to

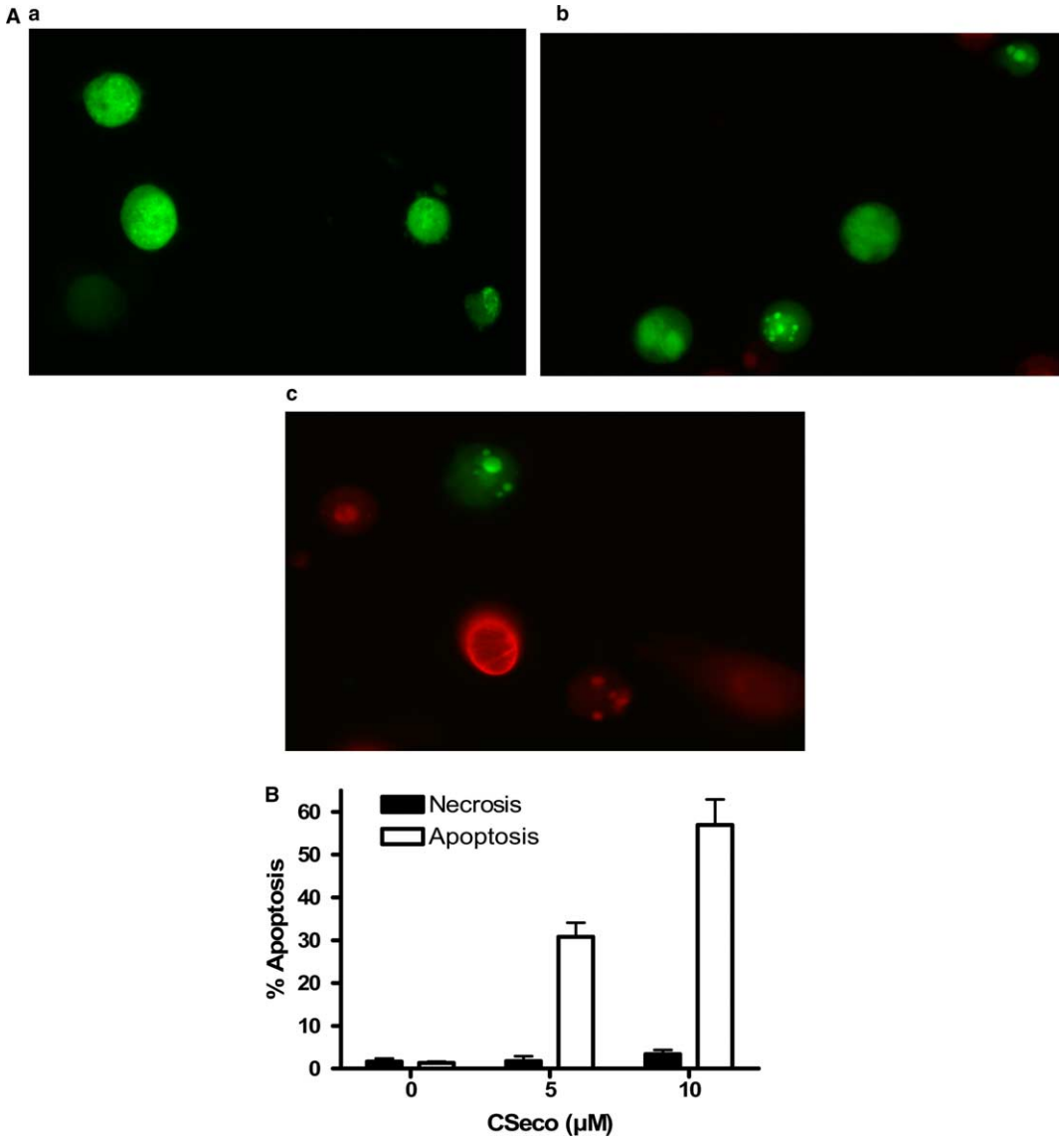

Fig. 5. (A) Induction of apoptosis in cardiomyocytes treated with CSeco: (a) Control cells (b) and (c) cardiomyocytes treated with 5 and 10 μM of CSeco (respectively) for 24 h. Apoptosis was detected using AO and EB morphological staining, and the fluorescence was observed using a fluorescence microscope. (B) Percentage of cardiomyocytes undergoing apoptosis/necrosis evaluated by AO/EB double staining following CSeco treatment after 24 h incubation.

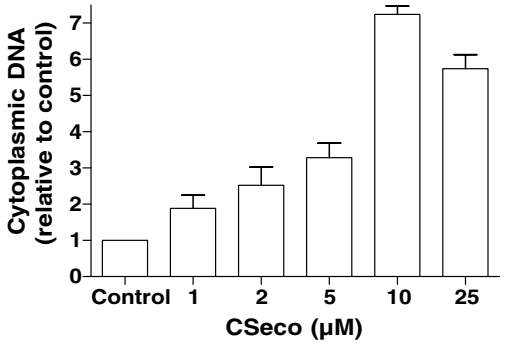

Fig. 6. Quantification of DNA fragmentation by measuring the cytoplasmic histone-associated DNA by sandwich ELISA. Cardiomyocytes are exposed to varying concentrations of CSeco for 24 h. Data are expressed relative to control values and are given as means ± SEM from five separate experiments.

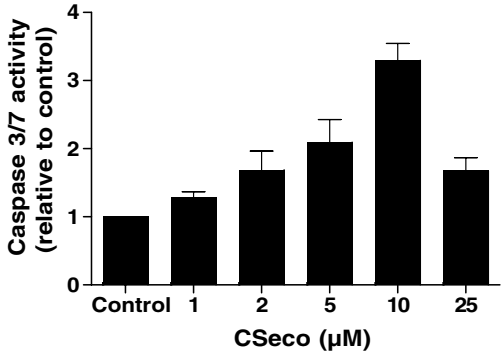

Fig. 7. Effect of CSeco on caspase-3/7 activity. Cardiomyocytes were treated with different CSeco concentrations. Results are expressed relative to control values. Data are means ± SEM of four separate experiments.

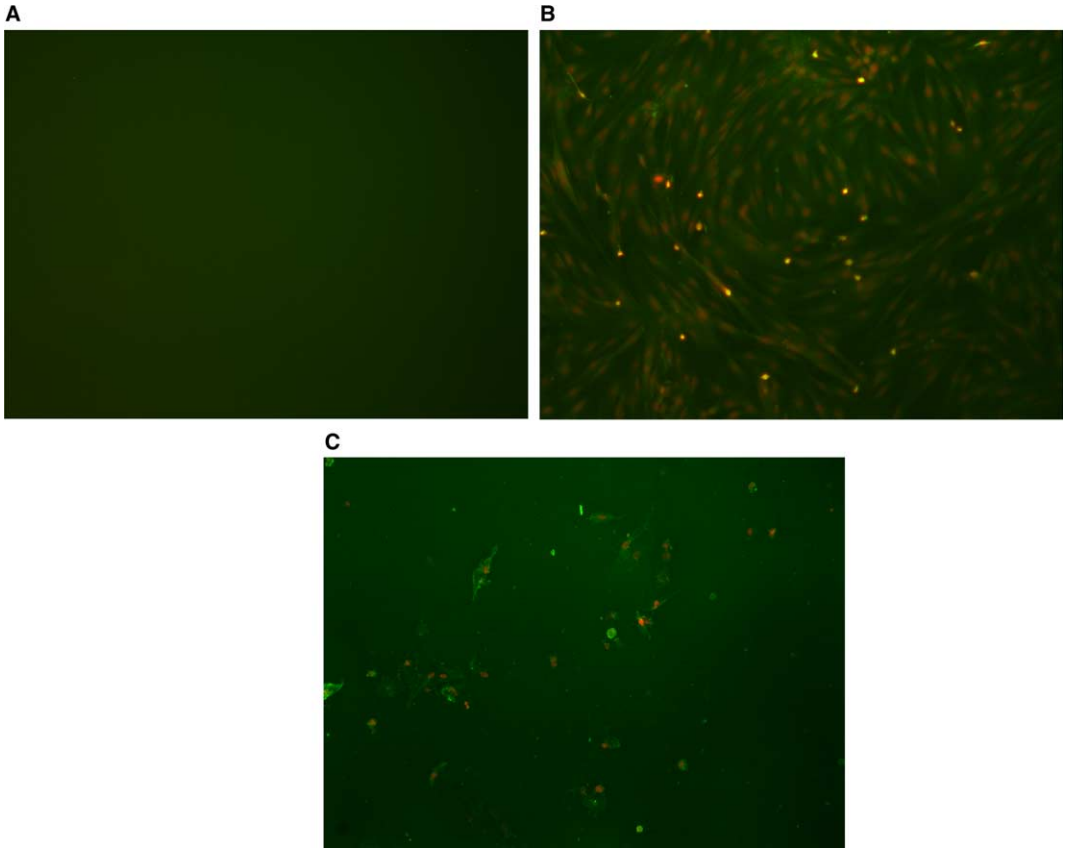

Fig. 8. Annexin V-FITC/propidium iodide staining photomicrographs: (A) untreated control cells; (B) and (C) cells treated with 5 and 10 μM CSeco for 24 h (respectively). Cardiomyocytes were stained using Annexin-V-FLUOS staining kit as described in Section 2.

peripheral tissues and organs, as postulated by Pryor and his colleagues [5,19]. Epidemiological studies indicate a link between ozone pollution and increased risk of myocardial infarction, coronary atherosclerosis, and pulmonary heart disease [10,11] and increased frequency of hospitalizations among the elderly [20]. Experimental studies also show that ozone exposure by inhalation causes sphering and increased osmotic fragility of erythrocytes [21], damage to the membranes and nuclei of myocardial fibers [22], and changes in brain serotonin levels [23], meaning that ozonation products formed at the air/lung interface can inflict damage to peripheral tissues and organs [24].

Pulfer and Murphy [7] have shown that exposure of lung surfactant to 2 ppm ozone produces three different types of oxysterols, namely, CSeco, 5-hydroperoxy-*B*-homo-6-oxa-cholestan-3β,7α-diol, and 5β,6β-epoxycholesterol [4,7]. 5-Hydroperoxy-*B*-homo-6-oxa-cholestan-3β,7α-diol and 5β,6β-epoxycholesterol have been shown to be cytotoxic to human bronchial epithelial cells [7]. According to Pulfer and Murphy [7], the yields of CSeco are lower than the other two sterols in ozone-exposed lung surfactant. The differences may not be significant when issues of cytotoxic potentials are considered. For instance, the concentration of CSeco at IC_{50} (8.9 ± 1.29 μM) is about half of that reported for hydroperoxy-*B*-homo-6-oxa-cholestan-3β,7α-diol (15 ± 8 μM). Therefore, the cytotoxicity observed in cardiomyocytes provide evidence for the biological effects of oxysterols produced in ozonation reactions.

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References

[1] Sadana, T., Dhall, K., Sanyal, S.N., Wali, A., Minocha, R. and Majumdar, S. (1988) Isolation and chemical composition of surface-active material from human lung lavage. Lipids 23, 551–558.
[2] Smith, L.L. (2004) Oxygen, oxysterols, ouabain, and ozone: a cautionary tale. Free Radic. Biol. Med. 37, 318–324.
[3] Wang, K., Bermudez, E. and Pryor, W.A. (1993) The ozonation of cholesterol: separation and identification of 2,4-dinitrophenylhydrazine derivatization products of 3β-hydroxy-5-oxo-5,6-secocholestan-6-al. Steroids 58, 225–229.
[4] Pulfer, M.K., Taube, C., Gelfand, E. and Murphy, R.C. (2005) Ozone exposure in vivo and formation of biologically active oxysterols in the lung. J. Pharmacol. Exp. Ther. 312, 256–264.
[5] Pryor, W.A., Wang, K. and Bermudez, E. (1992) Cholesterol ozonation products as biomarkers for ozone exposure in rats. Biochem. Biophys. Res. Commun. 30, 618–623.
[6] Wentworth Jr., P., Nieva, J., Takeuchi, C., Galve, R., Wentworth, A.D., Dilley, R.B., DeLaria, G.A., Saven, A., Babior, B.M., Janda, KD., Eschenmoser, A. and Lerner, R.A. (2003) Evidence for ozone formation in human atherosclerotic arteries. Science 302, 1053–1056.

[7] Pulfer, M.K. and Murphy, R.C. (2004) Formation of biologically active oxysterols during ozonolysis of cholesterol present in lung surfactant. J. Biol. Chem. 279, 26331–263338.
[8] Schroepfer Jr., G.J. (2000) Oxysterols: modulators of cholesterol metabolism and other processes. Physiol. Rev. 80, 361–554.
[9] Brown, A.J. and Jessup, W. (1999) Oxysterols and atherosclerosis. Atherosclerosis 142, 1–28.
[10] Koken, P.J., Piver, W.T., Ye, F., Elixhauser, A., Olsen, L.M. and Portier, C.J. (2003) Temperature, air pollution, and hospitalization for cardiovascular diseases among elderly people in Denver. Environ. Health Perspect. 111, 1312–1317.
[11] Ruidavets, J.B., Cournot, M., Cassadou, S., Giroux, M., Meybeck, M. and Ferrieres, J. (2005) Ozone air pollution is associated with acute myocardial infarction. Circulation 111, 563–569.
[12] Agrawal, S., Agarwal, M.L., Chatterjee-Kishore, M., Stark, G.R. and Chisolm, G.M. (2002) Stat1-dependent, p53-independent expression of p21(waf1) modulates oxysterol-induced apoptosis. Mol. Cell Biol. 22, 1981–1992.
[13] Giamalva, D.H., Church, D.F. and Pryor, W.A. (1986) Kinetics of ozonation. 5. Reactions of ozone with carbon-hydrogen bonds. J. Am. Chem. Soc. 108, 7678–7681.
[14] Janowski, B.A. (2002) The hypocholesterolemic agent LY295427 up-regulates INSIG-1, identifying the INSIG-1 protein as a mediator of cholesterol homeostasis through SREBP. Proc. Natl. Acad. Sci. USA 99, 12675–12680.
[15] Lim, H.K., Kang, H.K., Yoo, E.S., Kim, B.J., Kim, Y.W., Cho, M., Lee, J.H., Lee, Y.S., Chung, M.H. and Hyun, J.W. (2003) Oxysterols induce apoptosis and accumulation of cell cycle at G(2)/M phase in the human monocytic THP-1 cell line. Life Sci. 72, 1389–1399.
[16] Van Reyk, D.M. and Jessup, W. (1999) The macrophage in atherosclerosis: modulation of cell function by sterols. J. Leukoc. Biol. 66, 557–561.
[17] Zhang, Q., Powers, E.T., Nieva, J., Huff, M.E., Dendle, M.A., Bieschke, J., Glabe, C.G., Eschenmoser, A., Wentworth Jr., P., Lerner, R.A. and Kelly, J.W. (2004) Metabolite-initiated protein misfolding may trigger Alzheimer's disease. Proc. Natl. Acad. Sci. USA 101, 4752–4757.
[18] Babior, B.M., Takeuchi, C., Ruedi, J., Gutierrez, A. and Wentworth Jr., P. (2003) Investigating antibody-catalyzed ozone generation by human neutrophils. Proc. Natl. Acad. Sci. USA 100, 3013–3015.
[19] Pryor, W.A., Squadrito, G.L. and Friedman, M. (1995) The cascade mechanism to explain ozone toxicity: the role of lipid ozonation products. Free Radic. Biol. Med. 19, 935–941.
[20] Routledge, H.C., Ayers, J.G. and Townend, J.N. (2003) Why cardiologists should be interested in air pollution. Heart 89, 1383–1388.
[21] Menzel, D.B., Slaughter, R.J., Bryant, A. and Jauregui, H.O. (1975) Heinz bodies formed in erythrocytes by fatty acid ozonides and ozone. Arch. Environ. Health 30, 296–301.
[22] Rahman, I., Massaro, G.D. and Massaro, D. (1992) Exposure of rats to ozone: evidence of damage to heart and brain. Free Radic. Biol. Med. 12, 323–326.
[23] Skillen, R.G., Thienes, C.H., Cangelosi, J. and Strain, L. (1961) Brain 5-hydroxytryptamine in ozone-exposed rats. Proc. Soc. Exp. Biol. Med. 108, 121–122.
[24] Goldstein, B.D. (1978) The pulmonary and extrapulmonary effects of ozone. Ciba Found. Symp., 295–319.